Award Number: W81XWH-16-1-0200

TITLE: A Universal Platform for Identification of Novel Lung Cancer Biomarkers Based on Exosomes

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REPORT DATE: October 2017

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2017	Final	1 Jul 2016 - 30 Jun 2017

4. TITLE AND SUBTITLE
A Universal Platform for Identification of Novel Lung Cancer Biomarkers Based on Exosomes

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0200

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Leland Stanford Junior University
Palo Alto, CA

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Circulating tumor-derived extracellular vesicles (EVs) have emerged as a promising source for identifying cancer biomarkers for early cancer detection. However, the clinical utility of EVs has thus far been limited by the fact that most EV isolation methods are tedious, nonstandardized, and require bulky instrumentation such as ultracentrifugation (UC). Here, we report a size-based EV isolation tool called ExoTIC (exosome total isolation chip), which is simple, easy-to-use, modular, and facilitates high-yield and high-purity EV isolation from biofluids. ExoTIC achieves an EV yield ~4–1000-fold higher than that with UC, and EV-derived protein and microRNA levels are well-correlated between the two methods. Moreover, we demonstrate that ExoTIC is a modular platform that can sort a heterogeneous population of cancer cell line EVs based on size. Further, we utilize ExoTIC to isolate EVs from cancer patient clinical samples, including plasma, urine, and lavage, demonstrating the device's broad applicability to cancers and other diseases. Finally, the ability of ExoTIC to efficiently isolate EVs from small sample volumes opens up avenues for preclinical studies in small animal tumor models and for point-of-care EV-based clinical testing from fingerprick quantities (10–100 µL) of blood.

15. SUBJECT TERMS
None provided

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	13	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

Section	Page Number
Accomplishments	4
Impact	12
Products	12
Participants & Other Collaborating Organizations	12
Changes/Problems	13
Special Reporting Requirements	13

1. Accomplishments

What were the major goals of the project?

The objective of our research is to develop a universal exosome isolation and purification device to identify novel, exosome-based early diagnostic and prognostic biomarkers for lung cancer.

Aim 1 is to design, fabrication and optimization of the exosome total isolation chip (ExoTIC) device to isolate pure exosomes from culture media, urine, lavage, and human plasma.
Aim 2 is to identify lung cancer based biomarkers using exosomes, isolated with the ExoTIC from clinical plasma samples of patients.

What was accomplished under these goals?

Major activities:

We developed a size-based EV isolation tool called ExoTIC (exosome total isolation chip), which is simple, easy-to-use, modular, and facilitates high-yield and high-purity EV isolation from biofluids. ExoTIC achieves an EV yield ~4−1000-fold higher than that with UC, and EV-derived protein and microRNA levels are well-correlated between the two methods. Moreover, we demonstrated that ExoTIC is a modular platform that can sort a heterogeneous population of cancer cell line EVs based on size. Further, we utilized ExoTIC to isolate EVs from cancer patient clinical samples, including plasma, urine, and lavage, demonstrating the device' s broad applicability to cancers and other diseases. Finally, the ability of ExoTIC to efficiently isolate EVs from small sample volumes opens up avenues for preclinical studies in small animal tumor models and for point-of-care EV-based clinical testing from fingerprick quantities (10− 100 µL) of blood.

Specific objectives addressed:

Our team has worked on the design and fabrication of ExoTIC device and optimized the exosome isolation from different biofluids including plasma, urine, and lavage.

Significant results and key outcomes

Working principle of ExoTIC:

ExoTIC was specifically designed to simplify EV isolation in the research and clinical point of care settings. ExoTIC uses a simple filtration approach in which EV-containing clinical samples, including culture media, plasma, and urine (Figure 1a), are passed through a nanoporous membrane to enrich and purify intact EVs in the 30-200 nm size range. Free nucleic acids, proteins, lipids, and other small fragments are flushed out (Figure 1b) and concentrated EVs are collected from the filter membrane using a standard pipette. The EVs are then characterized using nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) for size, concentration, and morphology and then undergo downstream proteomic and transcriptomic analysis (Figure 1c). The fabrication process involves step-by-step assembly from laser-cut plastic layers, a polycarbonate track-etched nanoporous filter membrane, polyethersulfone (PES) layer, and cellulose pad. The plastic housing is secured with metal screws and nuts and a plastic ring-shaped gasket provides a leak-free seal. The cellulose pad prevents deformation of the filter membrane under the pressures generated by the syringe pump. The workflow of isolating EVs from culture media is shown in Figure 1d. A 10-mL sample solution, pre-filtered with a 0.22 µm PES syringe filter, is introduced continuously into the ExoTIC device *via* a 10-mL syringe using a syringe pump at a constant flow rate (5 mL/h). Once the sample has been concentrated down to 1 mL, the ExoTIC device is rotated by 180º so that the EVs are enriched at the end opposite to the inlet in order to minimize any potential sample loss. The concentrated EVs and any residual EVs bound to the filter membrane are recovered in the same tube, and the isolated EVs are then used for downstream physical characterization and molecular analysis.

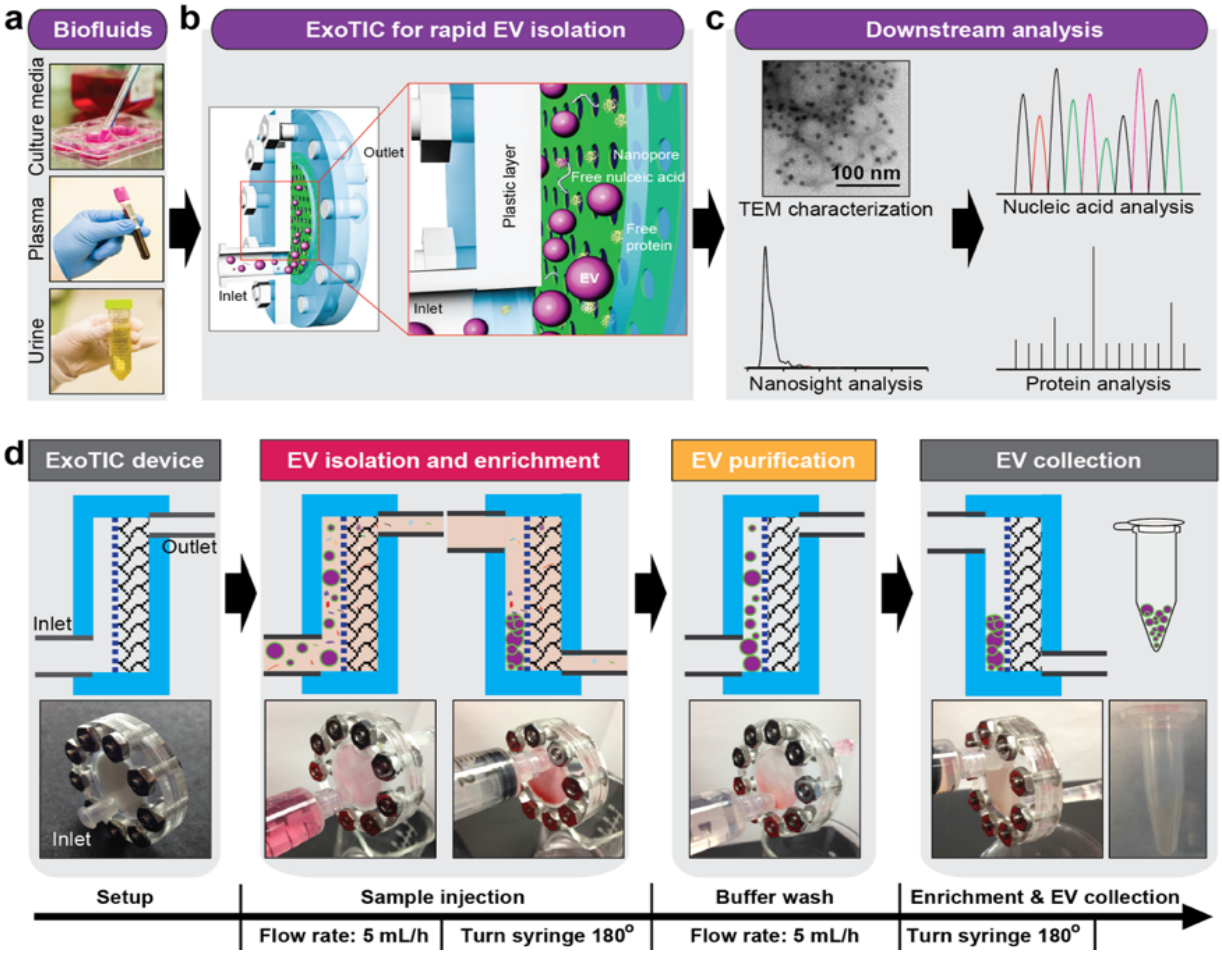

Figure 1. Schematic illustration of ExoTIC device for extracellular vesicle isolation. (a) Various biofluids can be processed for EV isolation in culture media, plasma, and urine. (b) The schematic illustration of size-based EV isolation using ExoTIC device. Intact EVs are enriched and purified at the filter, whereas the free proteins and nucleic acids are washed out. (c) Downstream analysis of EVs isolated from different clinical sample types for size, morphology and molecular contents. (d) Schematic process of EV isolation from sample-in to EV-out. Device operation includes: Isolation of EVs from cell culture media (5 mL in 1 hour), washing with PBS buffer (5 mL in 1 hour), and collection of ~ 200 µL of EV solution for subsequent analysis. Total operation time for 5-10 mL of sample in under 3 hours.

ExoTIC provides higher EV yields than UC and PEG precipitation:

ExoTIC was compared with UC and polyethylene glycol (PEG)-based precipitation with respect to yield, size, and morphology of EVs isolated from culture media. We found greater than 90% yield in enriched EVs compared to the initial culture media EV content. The size distribution of EVs was measured using nanoparticle tracking analysis (NTA) and their morphology and size were confirmed by scanning electron microscopy (SEM). As shown in Figure 2a, EVs isolated by UC had a unimodal peak centered at 91 nm by NTA and exhibited a size distribution of ~20-130 nm by SEM. The EVs purified by PEG precipitation formed aggregates and had multimodal peaks (Figure 2b). EVs isolated by ExoTIC had a unimodal peak at 99 nm by NTA (Figure 2c) with a size distribution of ~30-100 nm by SEM. ExoTIC also isolated EVs from culture media at a four-fold higher yield compared to UC (Figure 2d). The mean size of the EVs purified by all three methods was ~120 nm (Figure 2e). Figure 2f provides a comparison of the total number of EVs purified from different volumes (from 1 to 5 mL) of HCC827 cell culture medium using ExoTIC, with the expected linear correlation between EV number and media volume. The TEM

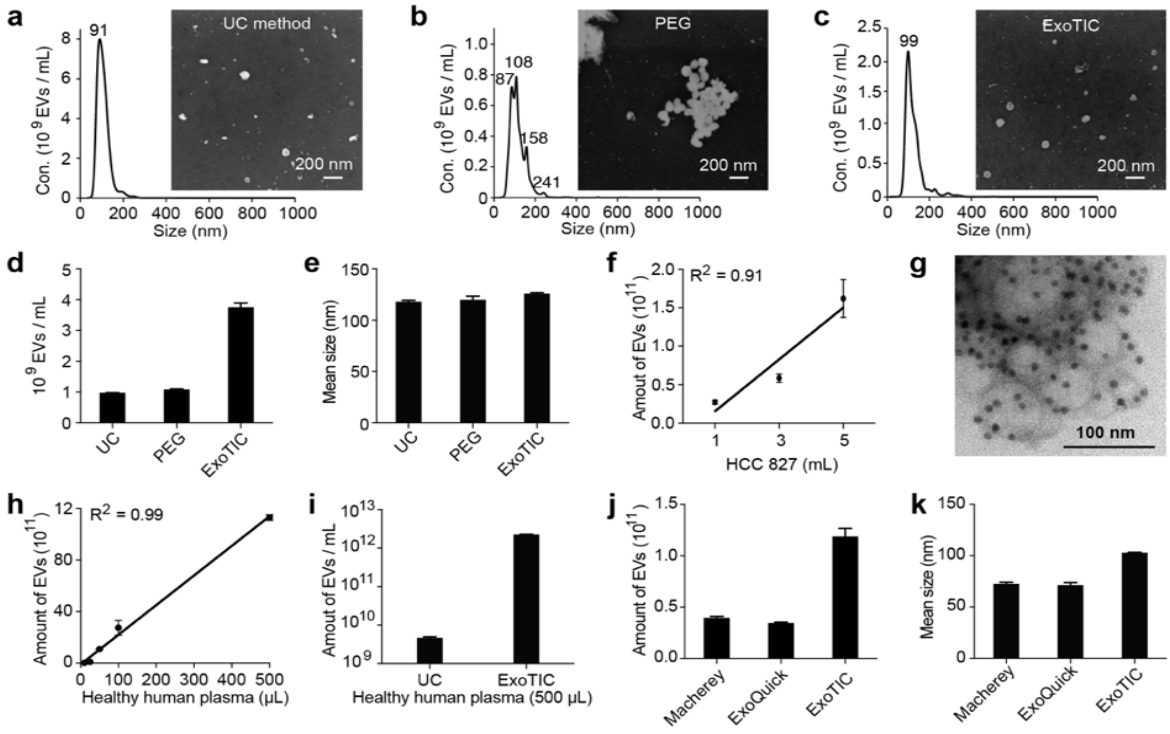

Figure 2. Physical properties of EVs isolated by different methods. NTA and SEM analysis of EVs isolated by (a) UC, (b) PEG-based precipitation, and (c) ExoTIC. (d) Yield comparison of EVs purified from HCC827 lung adenocarcinoma cell culture medium by the ExoTIC device (5 mL), ultracentrifugation (60 mL), and PEG (5 mL). (e) Mean size of EVs purified by the three methods as determined by NTA (NanoSight NS300). (f) Total quantity of EVs purified from different volumes of HCC827 cell culture medium using the ExoTIC device. (g) TEM image of EVs isolated from cell culture media (HCC827 cell line) using the ExoTIC device, immunogold labeled for CD63 (dark spots). (h) Demonstration of the ExoTIC device's ability to isolate EVs from plasma volumes as low as 10 µL up to 500 µL. (i) Yield comparison between UC and ExoTIC device of EVs isolated from 500 µL of plasma from healthy human donors. Comparison of three different isolation methods with respect to (j) yield and (k) mean size (as determined by NTA) of EVs isolated from 100 µL plasma. (Mean +/- s. d. n=5). Mean size refers to the average size of the EVs in the size distribution. Mean size values are automatically generated in the Nanosight report.

image in Figure 2g shows EVs with the hallmark cup-shaped artifact. These results demonstrate that the ExoTIC device can efficiently isolate EVs with a wide size distribution while avoiding the use of polymer contaminants.

We then compared the performance of ExoTIC with UC and PEG precipitation in isolating EVs from the plasma of healthy patients. We first investigated the ability of the ExoTIC device to process low volumes (10 to 500 µL) of plasma (Figure 2h). When compared to UC purification of the same volume, the EV yield purified from 500 µL of healthy human plasma by the ExoTIC device was ~1,000 times higher (Figure 2i). When compared with commercial PEG precipitation kits (ExoQuick and Macherey), the ExoTIC device achieved three to four-fold higher EV yields (Figure 2j). The mean size of EVs isolated by ExoTIC was ~30% larger than that isolated by the other two commercial kits (Figure 2k), which may be due to the isolation methods.

Evaluation of EV microRNAs by ExoTIC and UC:

EV microRNAs are a potential source of cancer biomarkers for early cancer diagnosis. Using conditioned culture media from lung cancer cell lines (HCC827 and H1650) as a proof-of-concept, we compared ExoTIC- and UC-isolated EVs with respect to miRNA profiles. We first characterized the quantity and morphology of the EVs and subsequently analyzed their microRNAs expression levels. ExoTIC again isolated >4-fold higher yields of EVs from conditioned culture media compared to UC (Figure 3a), and 6-fold higher yields of EV-derived microRNA compared to UC (Figure 3b). MicroRNA expression levels between ExoTIC and UC

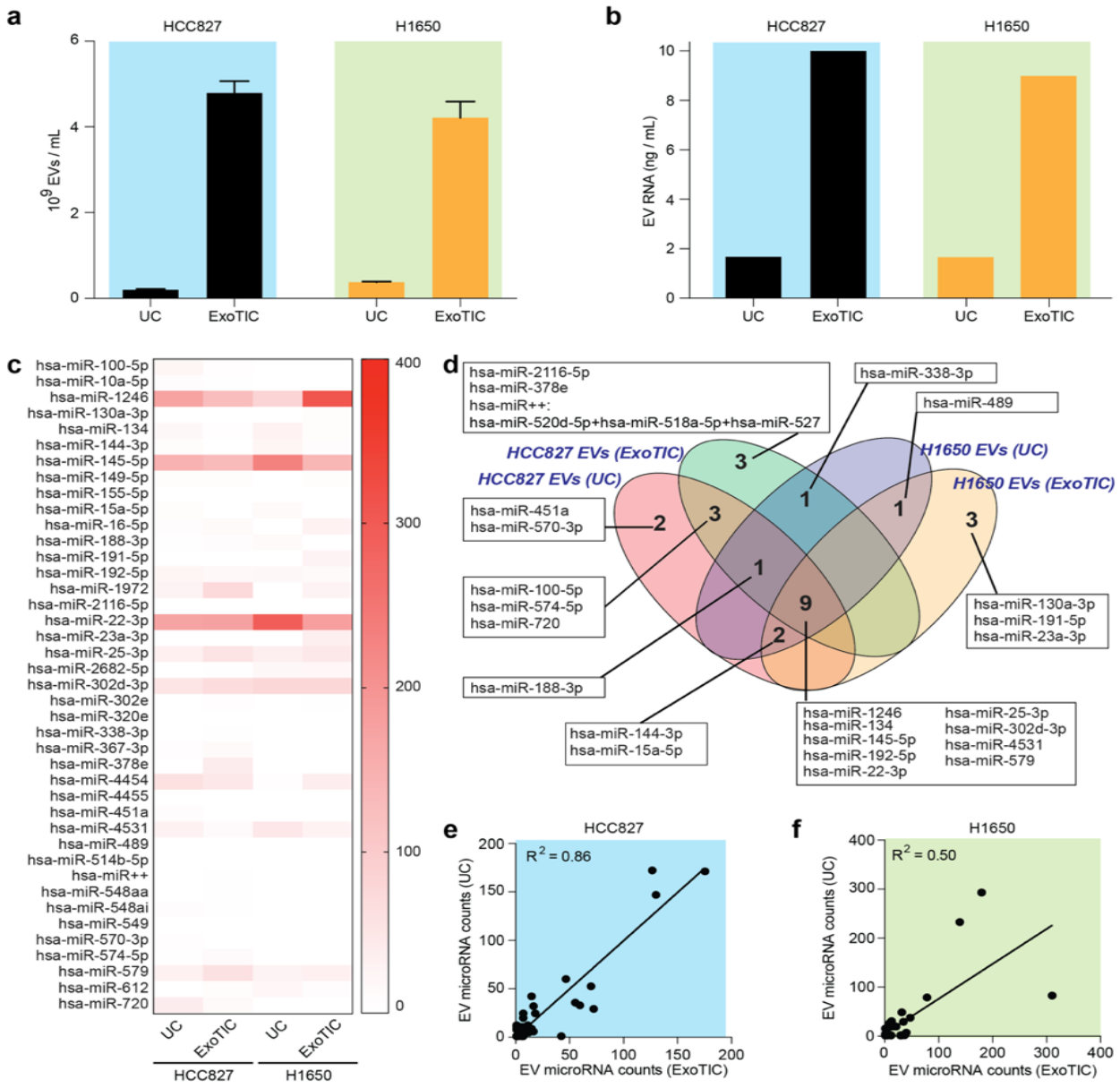

Figure 3. Evaluation of microRNA extracted from EVs isolated ExoTIC with UC. (a) EVs isolated from UC (60 mL) and ExoTIC (10 mL) in two cancer cell lines, HCC827 and H1650 (Mean +/- standard deviation, n=5). (b) EV RNA extracted from EVs isolated in the same culture media using UC and ExoTIC. (c) Heat-map of the top forty EV microRNAs. (d) Venn diagram of identified EV microRNAs between UC and ExoTIC. Venn diagram indicates overlapping and non-overlapping microRNAs between the two methods and in two cell lines. (e) and (f) linear correlation of EV microRNA expression levels between the two methods in two cell lines. R^2 represents Pearson correlation coefficient between two methods.

were further analyzed by absolute quantitation of over 800 distinct microRNAs using fluorescent tags and digital imaging (Nanostring™) (Figure 3c). The forty most highly expressed microRNAs were common (100% overlap) to both EV isolation methods. The Venn diagram in Figure 3d for two lung cancer cell lines shows both overlapping and non-overlapping EV microRNAs (25 most

highly expressed microRNAs) between ExoTIC and UC. For the HCC827 cell line, 13 microRNAs were found to be highly expressed in both UC and ExoTIC methods. Four

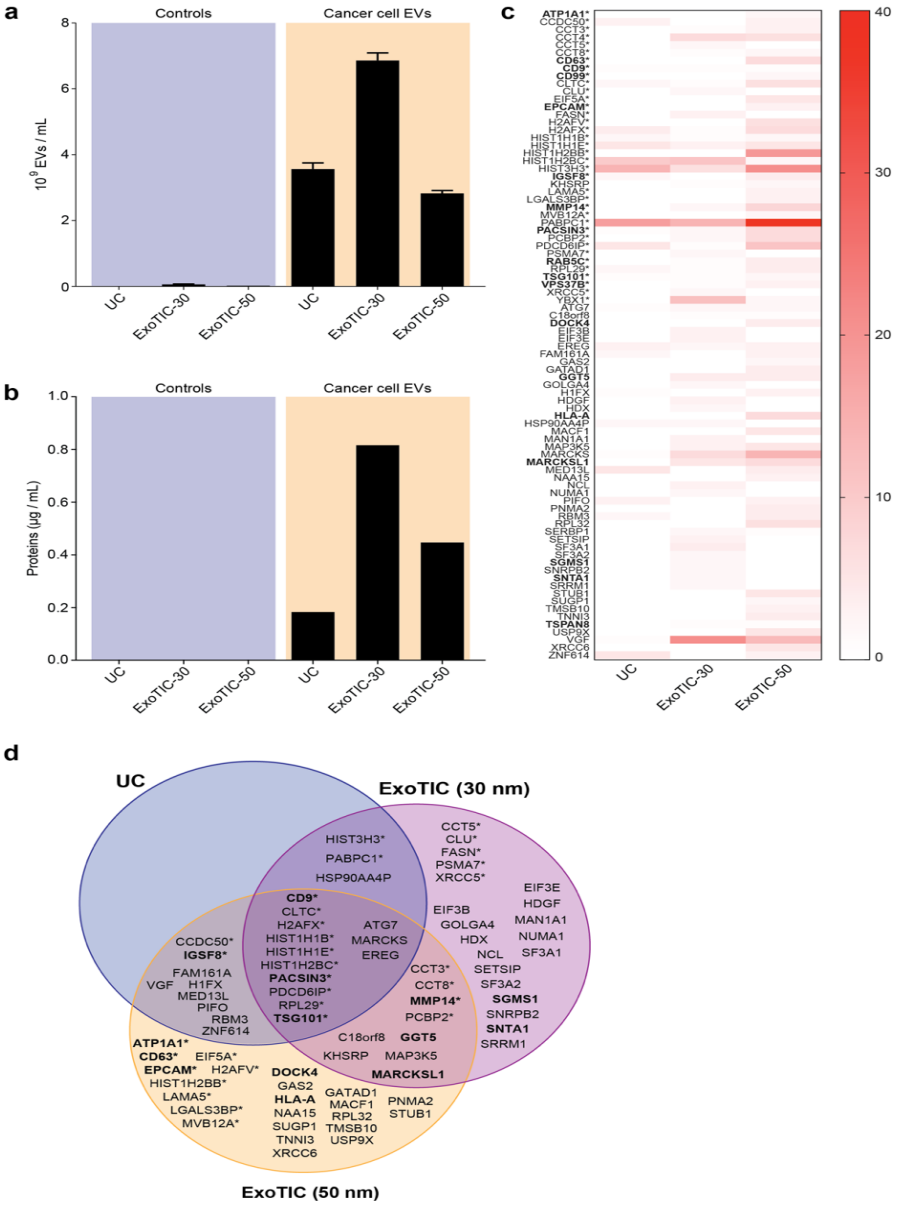

Figure 4. Evaluation of EV protein composition. (a) Comparison of EV yield isolated from culture media of 22Rv1 cells in serum-free media and controls (without 22Rv1 cells in serum-free media) using UC, ExoTIC-30 (filter pore: 30 nm), and ExoTIC-50 (filter pore: 50 nm), respectively. (b) Comparison of EV protein yield from EV samples in (a). (c) Number of peptides identified by LC-MS/MS of EVs from 22Rv1 cells isolated by UC, ExoTIC-30, and ExoTIC-50, respectively. (d) Venn diagrams of proteins identified from EVs using different methods. For the proteins shown, no peptides were observed in control samples (media with and without EVs). *Indicates previously identified in EVs from prostate cancer cell lines (www.exocarta.org). **Bold** indicates membrane protein.

microRNAs demonstrated high expression only in UC, and three microRNAs and one microRNA cluster demonstrated high expression only in ExoTIC. For the H1650 cell line, three microRNAs demonstrated high expression in ExoTIC, and 12 microRNAs showed high expression by both UC and ExoTIC. Nine of the highly-expressed microRNAs were common to both cell lines and

both isolation methods. Of these, hsa-miR-1246 and hsa-miR-134 have been reported to be highly correlated with lung cancer. Figure 3e and 3f demonstrates the linear correlation of EV microRNA expression levels between the two methods in the two cell lines. The correlation coefficients (R^2) between ExoTIC and UC microRNA counts are 0.86 and 0.50 for cell lines HCC827 and H1650, respectively. The difference in correlation coefficients between the two cell lines is due to cell line-specific differences in EV size and density.

Although ExoTIC and UC methods showed similar microRNA profiles for the top forty EV microRNAs, we also observed differences in EV-associated microRNAs in conditioned media from cancer cell lines grown in culture. For example, certain microRNAs were more highly expressed in EVs isolated by ExoTIC compared to UC, and vice versa. These findings are supported by previous studies, which demonstrated stoichiometric differences in microRNA contents among different EV populations and between different methods. These differences in microRNA expression profiles can be attributed to the different separation mechanisms used by the two methods, which result in non-identical EV populations, as UC separates EVs based on *density* while ExoTIC separates EVs based on *size*.

Evaluation of EV protein expression in ExoTIC and UC

EVs derived from cancer cells carry a payload of proteins that reflect proteins secreted and shed by the tumor. Isolated EV proteins are a rich source of biomarkers that could allow stratification of cancer patients into low-risk and high-risk groups for treatment. As a proof-of-concept, we demonstrated isolation of EVs and EV proteins from prostate cancer cell lines. Using liquid chromatography-mass spectrometry (LC/MS), we compared the ExoTIC and UC methods with respect to protein expression in EVs isolated from conditioned culture media from 22Rv1 prostate cancer cells. For 48 hours prior to EV collection, we incubated cells in serum-free culture media to ensure that the proteins we detected were EV-specific and free of contaminating serum proteins and bovine EVs. To study the effect of EV size on protein profile, we isolated EVs using ExoTIC devices with two different pore sizes (30 nm and 50 nm) and traditional UC. As with the lung cancer cell lines discussed previously, substantially more prostate cancer cell line EVs were isolated by either ExoTIC device than by UC (Figure 4a). Proteins were isolated from each of the EV preparations and measured as shown in Figure 4b. Our data presented in Figure 4c and 4d, of the 84 EV-derived proteins that we identified in total, 29% (25/84), 62% (52/84), and 75% (63/84) were identified in EVs from UC, ExoTIC-30nm, and ExoTIC-50nm, respectively. Thirty-seven of these proteins have previously been reported to be associated with this cancer cell line (Exocarta Database, www.exocarta.org). Of the 37 proteins, 29% (11/37) were common to UC and ExoTIC-30nm, 37% (14/37) were common to UC and ExoTIC-50nm, and 54% (20/37) were common to ExoTIC-30 and ExoTIC-50. The 30-nm and 50-nm pore-size ExoTIC devices accounted for most of prostate cancer-associated EV proteins that were detected. Our data show that we could detect higher number of EV protein using ExoTIC when compared to UC.

Different ExoTIC pore sizes (30 and 50 nm) may yield different EV populations, resulting in distinct protein contents. The lack of 100% overlap between the 30 nm and 50 nm isolation is likely due to two effects, which includes differences in pressure drop in ExoTIC devices and the sampling of the mass spectrometer. The first possible explanation for this difference is that achieving the same flow rate (5mL/hr) in both devices requires ~5-fold higher operating pressure in the 30nm device than in the 50nm device ($\Delta P \sim 1/r^3$). These higher pressures may have changed overall filtering characteristics of the device for that specific flow rate range and pore size, which may have resulted in differences in isolating EVs. This is an indication that the flow optimization must be done separately for each filter size over the changing flow resistance. The second likely explanation for the lack of overlap of this subset of proteins is due to the sampling of the mass spectrometer and not every protein being comprehensively measured in

LC-MS run (a limitation of MS). In a typical shotgun, LC-MS/MS experiment, the mass spectrometer selects peptides as they elute from the LC for fragmentation in the MS. The number of peptides that can be fragmented in a complex mixture exceeds the speed of the mass spectrometer, therefore not all the same peptides are sampled from run-to-run.

ExoTIC as a modular platform for size-based sorting of EVs

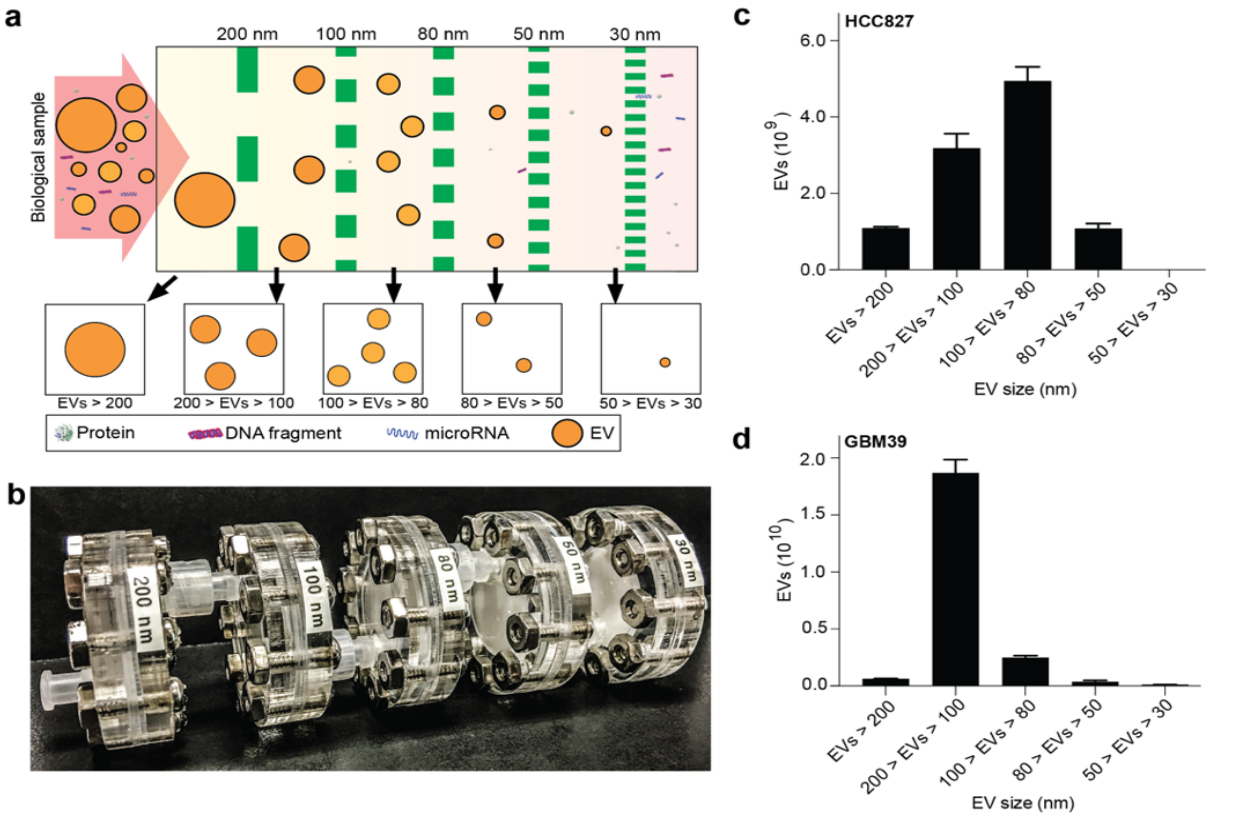

Figure 5. Size based isolation of EVs using the ExoTIC device. (a) Design schematic and (b) actual modular ExoTIC device, with five different membrane pore sizes that connect in series for differential isolation of EVs by using the same input sample. Quantity of EVs isolated at each size cutoff in the series from (c) HCC827 culture media, and (d) GBM39 culture media. (Mean +/- standard deviation, n=5).

Cells release EVs in a broad range of sizes, with size-associated differences in biomolecular content. To address the need to sort different-sized EVs from the same sample, we designed ExoTIC as a modular unit such that several ExoTIC devices, each with a different membrane pore size (*e.g.* 200, 100, 80, 50, and 30 nm), can be connected in series to isolate EVs at several specific, narrow size ranges (Figure 5a, 5b). To validate our design, HCC 827 cell culture media was injected into a series of ExoTIC modules with pore sizes 200, 100, 80, and 50 nm. Retentates containing isolated EVs were separately collected from each ExoTIC module in the series and analyzed by NTA, confirming that progressively smaller EVs are captured at membranes of successively smaller pore sizes. The modular ExoTIC arrangement was also used to compare differences in EV amount and size between two cancer cell lines, HCC 827 and GBM 39 (glioblastoma) (Figure 5c and 5d). We found distinctly different EV sizes and quantities between these two cell lines. The histogram of mode EV size at each filter cutoff corresponds well to the size distribution of EVs, showing that the modular system can sort different-sized EV subpopulations without affecting the overall size distribution, as might occur if there was preferential loss of EV yield at a filter size. The mode size of the exosomes that pass through the filter decreased as the filter pore size became smaller. The mode size and number

of exosomes in a specific size range showed dependence on the cell lines. Thus, the modular ExoTIC system enables size-fractionation of EVs for size-specific molecular analysis.

EV Isolation from NSCLC Biological Fluids

a

Patient #	Final diagnosis	Clinical samples		
		Plasma	Lavage	Urine
19	Stage I NSCLC	0.4 mL	2.5 mL	10 mL
20	Stage III NSCLC	0.5 mL	2.0 mL	10 mL
25	Stage III NSCLC	0.2 mL	3.0 mL	10 mL
97	Stage I NSCLC	0.5 mL	2.4 mL	10 mL
111	Stage I NSCLC	0.6 mL	2.0 mL	10 mL

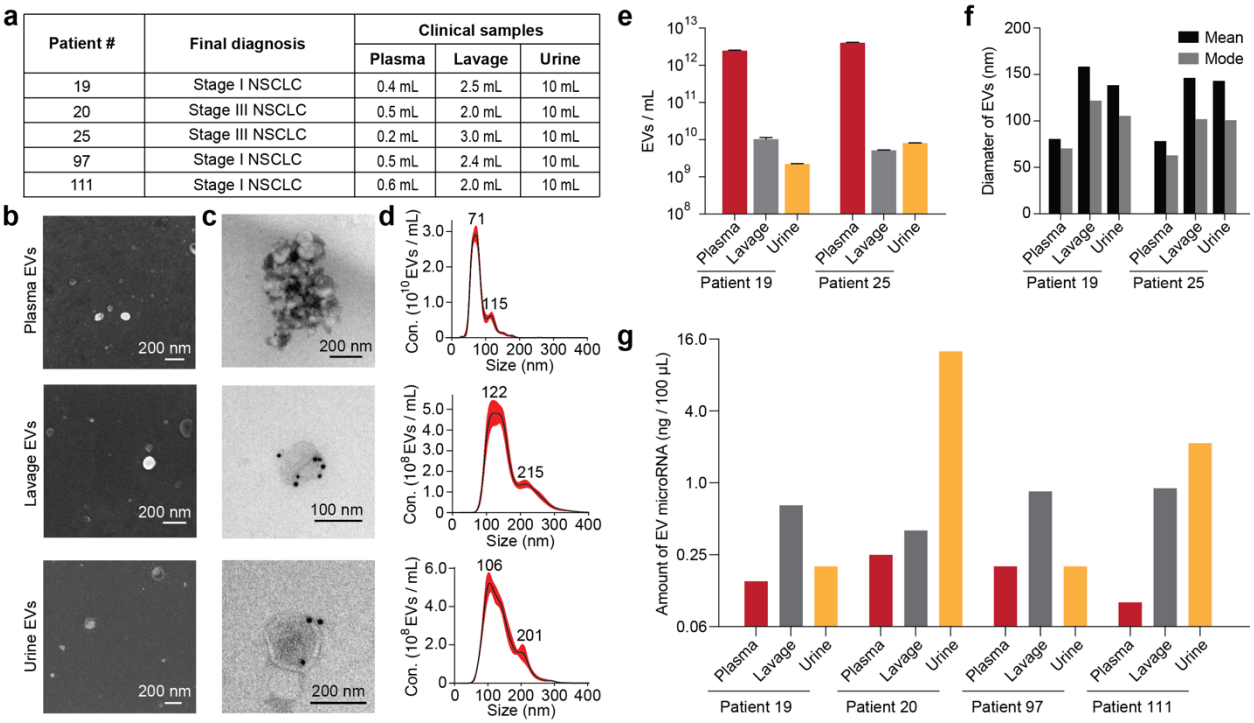

Figure 6. Isolation and characterization of EVs from different biofluids of cancer patients. (a) Patient information and sample volume used for plasma, bronchoalveolar lavage (BAL) and urine to isolate EVs. EVs of patient 19 by fluid type and (b) SEM, (c) TEM with immunogold labeling for CD63 (GNP dia. 10 nm), and (d) nanoparticle tracking analysis of concentration (e) Total quantity and (f) mean size and mode size of EVs isolated from the plasma, lavage, and urine of Patient 19 and 25. (g) The amount of EV microRNAs extracted from the plasma, lavage, and urine of four lung cancer patients.

Next, we evaluated the efficiency and reproducibility of EV purification from limited clinical samples - including blood plasma, urine, and lung bronchoalveolar lavage (BAL) fluid from patients with non-small cell lung cancer (NSCLC) (Figure 6a). BAL is the recovery of fluid instilled into the airway during bronchoscopy and is routinely used to evaluate and diagnose lung cancer and other lung diseases. We successfully isolated EVs from these clinical samples and characterized their size and morphology by SEM (Figure 6b) and TEM (Figure 6c). The morphology and size distribution of the particles, as well as the presence of the EV-specific surface marker CD63, confirmed that the particles we had isolated with ExoTIC device were in fact EVs (Figure 6c, and 6d). It is noteworthy that EVs from plasma were smaller and more abundant than EVs from BAL and urine (Figure 6e and 6f). Quantification was performed on total RNA isolated from EVs in the plasma, BAL, and urine samples of four patients with lung cancer. Although plasma yielded the most EVs, RNA quantities were in fact lowest in plasma EVs and highest in urine EVs (Figure 6g).

Describe the Regulatory Protocol and Activity Status (if applicable).

(c) Clinical Smaple Use Regulatory Protocols

TOTAL PROTOCOL(S): 3

PROTOCOL: Stanford University

Protocol [ACURO Assigned Number]: **LC150650**

Title: A Universal Platform for Identification of Novel Lung Cancer Biomarkers Based on Exosomes

Status: responses are being reviewed by Stanford University

What do you plan to do during the next reporting period to accomplish the goals and objectives?
Not applicable, as this is the final report.

2. Impact: Development of new exosome isolation device.

3. Products

Journal Article:

1. Liu F, Vermesh O, Mani V, Ge TJ, Madsen SJ, Sabour A, Hsu EC, Gowrishankar G, Kanada M, Jokerst JV, Sierra RG, Chang E, Lau K, Sridhar K, Bermudez A, Pitteri SJ, Stoyanova T, Sinclair R, Nair VS, Gambhir SS, Demirci U., "The exosome total isolation chip." *ACS Nano*. 2017 Nov 1. doi: 10.1021/acsnano.7b04878.

Patents:

1. Title: Exosome-Total-Isolation-Chip (ExoTIC) Device for Identification of Exosome-based Biomarkers.
   Details: Stanford Office of Technology Licensing, Docket S16-048.
   Description: We reported a platform to selectively isolate exosomes for exosome-based assays.

4. Participants & Other Collaborating Organizations Contribution to Project:

Name:	Utkan Demirci
Project Role:	PI
Research Identifier:	utkandemirci (NIH agency login)
Month worked:	0.36 calendar months in 1 year
Contribution to Project:	Supervision of project at Stanford.
Nearest person:	Lars Steinmetz
Project Role:	Co-I
Research Identifier:	2220295
Month worked:	0.38 calendar months in 1 year
Contribution to Project:	Dr. Steinmetz is responsible for the genome and transcriptome analysis.
Name:	Sanjiv Sam Gambhir
Project Role:	Collaborator
Research Identifier:	gambhir2
Month worked:	effort as needed
Contribution to Project:	Dr. Gambhir will be responsible for the overall planning, design, and implementation of the portion of the project that will involve the generation of clinical samples of patients at Stanford University.
Name:	Fei Liu
Project Role:	Postdoc
Research Identifier:	none

Month worked: 6.6 calendar months
Contribution to Project: Dr. Liu is responsible for developing microfluidic devices and doing validation studies.

Person: Alessandro Tocchio
Project Role: Postdoc
Research Identifier: none
Month worked: 3.6 calendar months
Contribution to Project: This postdoc is responsible for sample collection and analysis.

Person: Rami El Assal
Project Role: Research Scientist
Research Identifier: relassal
Month worked: 6 calendar months
Contribution to Project: This research scientist is responsible for sample collection and analysis.

Person: Woei-Jong Liu
Project Role: Research Scientist
Research Identifier: none
Month worked: 6 calendar months
Contribution to Project: This research scientist is responsible for sample collection and analysis.

5. Changes/Problems

Actual Problems or delays and actions to resolve them

None

6. Special Reporting Requirements

None